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Sicca, Federica; Neppelenbroek, Sam; Huckriede, Anke

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## Effector mechanisms of influenza-specific antibodies: neutralization and beyond

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REVIEW



## Effector mechanisms of influenza-specific antibodies: neutralization and beyond

Federica Sicca, Sam Neppelenbroek and Anke Huckriede

Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

### ABSTRACT

**Introduction:** Antibodies directed against influenza virus execute their protective function by exploiting a variety of effector mechanisms. Neutralizing antibodies have been thoroughly studied because of their pivotal role in preventing influenza virus infection and their presence in host serum is correlated with protection. Influenza antibodies can also exploit non-neutralizing effector mechanisms, which until recently have been largely overlooked.

**Areas covered:** Here, we discuss the antibody response to influenza virus in its entire breadth. Neutralizing antibodies mostly target variable epitopes on influenza surface proteins and interfere with virus binding, fusion, or egress. Non-neutralizing antibodies instead usually target conserved epitopes which can be located on surface as well as internal proteins. They drive viral clearance via interaction of their Fc region with components of the innate immune system such as immune effector cells (e.g. NK cells, macrophages) or the complement system.

**Expert commentary:** Recent research has unraveled that influenza-specific antibodies target multiple proteins and make use of diverse effector mechanisms. Often these antibodies are cross-reactive among virus strains of the same subtype or even between subtypes. As such they are induced early in life and are boosted by regular encounters with virus or vaccine. Designing strategies to optimally exploit these pre-existing antibodies may represent the key for the development of new broadly protective influenza vaccines.

### ARTICLE HISTORY

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### KEYWORDS

ADCC; ADE; ADCP; broadly neutralizing antibodies; complement; FcγR; immunoglobulin; influenza; non-neutralizing antibodies; universal vaccine

### 1. Introduction

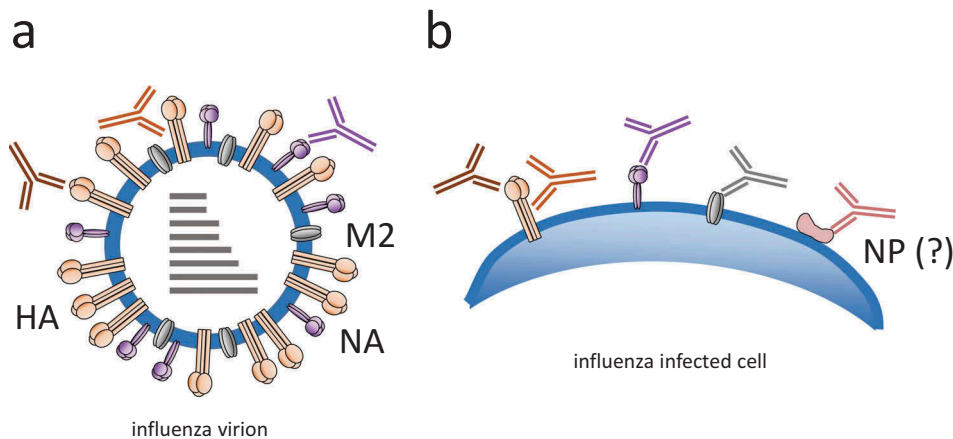
Influenza is a major public health problem as it is associated with high rates of morbidity and mortality worldwide. Type A and type B influenza viruses cause this respiratory tract infection which is characterized by sudden onset of symptoms such as high fever, headache, myalgia, malaise, and a sore throat [1]. The majority of individuals infected with influenza virus recover without medical attention. However, young children, the elderly, and chronically ill people are groups at risk for developing severe illness, which can be fatal.

Influenza viruses are enveloped viruses which belong to the Orthomyxoviridae family. The two clinically relevant genera of the Orthomyxoviruses are influenza virus A and influenza virus B. Among the influenza A viruses, different subtypes can be distinguished based on the subtypes of hemagglutinin (HA) and neuraminidase (NA) [2]. So far, 18 different subtypes of the HA protein (H1-H18) and 11 different subtypes of the NA protein (N1-N11) have been identified [3]. Influenza B viruses form two lineages, the B/Victoria/2/87-like lineage and the B/Yamagata/16/88-like lineage, but are not further divided into subtypes [4]. The genome of influenza viruses is composed of single-stranded RNA organized in separate segments [5]. This genome make-up enables rapid evolution of influenza virus since replication of RNA is highly error-prone resulting in frequent point mutations. Moreover, the segmented nature of the genome enables the swap of entire

segments if two different viruses infect a given cell simultaneously. This phenomenon is observed mainly for influenza A viruses which circulate in a number of animal species, in contrast to influenza B viruses which are with few exceptions restricted to humans. If point mutations or segment swap affect the HA glycoprotein, the major antigen of influenza virus, they can lead to antigenic drift and antigenic shift, respectively, thus enabling the virus to escape immune responses evoked by prior infection or vaccination [6,7].

Cellular as well as humoral immune mechanisms are involved in the defense against influenza virus infection. Cellular immunity, in particular CD8+T-cell-mediated immunity, mainly targets internal viral proteins like nucleoprotein (NP), matrix protein 1 (M1), or the polymerase proteins (PA, PB1, PB2), which are rather conserved across different influenza virus strains [8,9]. In contrast, influenza-specific antibodies mainly target the viral surface proteins, HA and NA, of which the most exposed parts are highly variable. Yet, antibodies directed against conserved regions on the HA head and particularly the HA stem as well as antibodies against a range of other more conserved viral proteins can also be found in recently infected individuals (Figure 1) [10]. These antibodies have lately been shown to play an important role in protection against influenza virus infection [9].

Antibodies directed against influenza virus make use of a variety of effector mechanisms [11,12]. Antibody-mediated neutralization is the most important mechanism and can



**Figure 1.** Targets of influenza-specific antibodies. (a) Antibody-targeted protein domains on virions. Antibodies may target the head or stem region of HA or the NA protein. (b) Antibody-targeted protein domains on infected cells. On the membrane of infected cells, antibodies can access the HA head and stem, NA, M2 and possibly NP.

prevent influenza virus infection of host cells. The ability of antibodies to neutralize influenza virus is correlated with protection and has, therefore, been studied thoroughly [13,14]. The majority of neutralizing antibodies is directed against the highly variable head region of the HA molecule. As such the activity of these antibodies is highly strain-specific. Besides neutralization, antibodies directed against influenza virus can also make use of effector mechanisms, which do not interfere with the initial infection steps but elicit their protective function later in the viral life cycle [15]. More complex immune interactions are needed for the non-neutralizing antibodies to work. In contrast to neutralizing antibodies which target the variable HA molecule, non-neutralizing antibodies can be directed to a variety of influenza virus proteins. While recognition of the target protein is mediated by the variable parts of the antibody molecules, their biological function is determined by the constant parts of the heavy chains, the Fc part. Accordingly, the class and subclass of the antibodies are very important for the type of effector functions they can engage in.

Neutralizing antibodies are highly effective in protecting from influenza infection but at least the classic neutralizing antibodies detected in hemagglutination inhibition assays are not effective against newly emerging influenza virus strains since they are strain-specific. Non-neutralizing antibodies, on the other hand, often target more conserved epitopes. Although they cannot prevent initial infection, they may provide a certain level of protection also against newly emerging influenza virus strains [16,17]. A thorough understanding of the different types of influenza-specific antibodies, their targets and their working mechanisms might thus be useful for the design of broadly protective vaccines. This review will give an overview of antibody-mediated effector mechanisms involved in protection against influenza virus.

## 2. Working mechanisms of neutralizing antibodies

The HA and NA glycoproteins are the most immunogenic proteins of influenza A and B and, therefore, represent the main target for neutralizing antibody responses elicited by

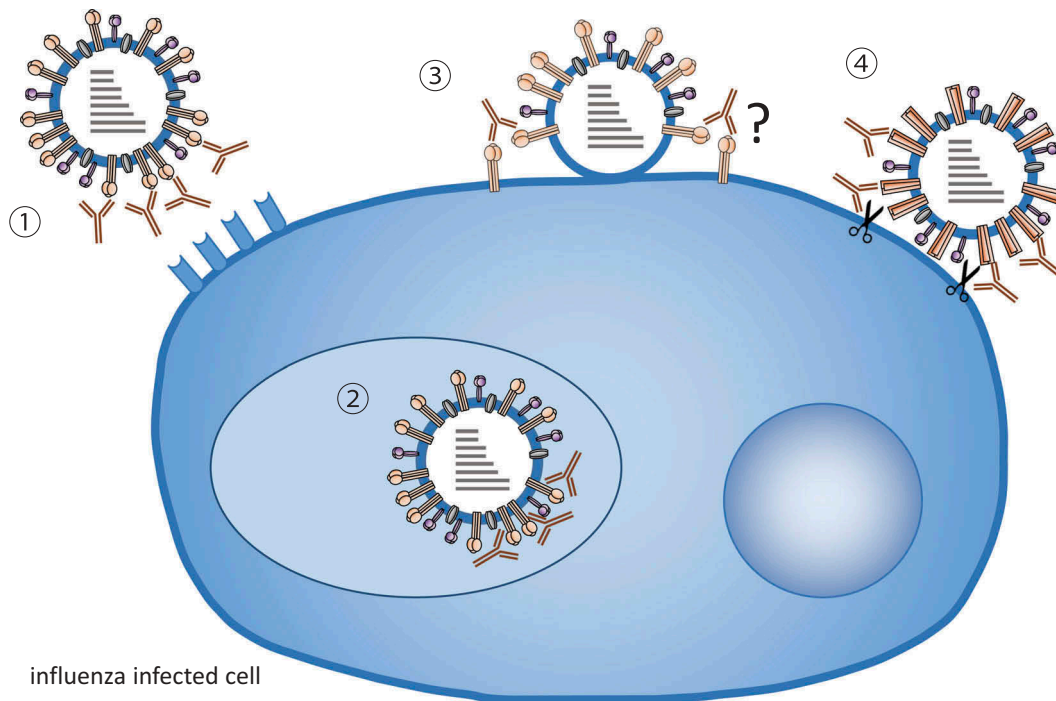
infection or vaccination. Most antibodies reacting with influenza virus are directed against the easily accessible globular head of HA [18]. The globular head also harbors the receptor-binding domain and engages with sialic acid residues on target cells [19]. After endocytosis of the virus, the stem region of HA mediates the low pH-triggered fusion of the viral and the endosomal membrane by which the viral genome gets access to the cytosol [10,19–21]. NA on the other hand is a tetramer that has enzymatic function; it removes sialic acid residues from the surface of infected cells, thereby allowing the release of virions after the budding process.

Neutralizing antibodies can prevent binding of the virus to the sialic acid receptor, can hamper the fusion process, or can interfere with the release of newly formed viral particles (Figure 2). Not each of these processes is neutralizing in the sense that initial entry of the target cell is prevented, however, all reduce virus spread in the first infection cycle. Traditional hemagglutination inhibition assays measure only those antibodies which interfere with binding of the virus to the host cell. Accordingly, neutralizing antibodies targeting other processes have long been overlooked.

### 2.1. Neutralizing antibodies targeting the HA head

Antibodies against the HA head usually result in strain-specific protection since they bind highly variable epitopes. It has been shown that these antibodies interfere with the entry of influenza virus by inhibiting the binding between HA and sialic acid. As such they represent the main type of antibodies detected in a classical hemagglutination inhibition assay [22–25]. Neutralizing antibodies directed against the HA head nevertheless have also been shown to be able to interfere with the egress of virions from an infected cell [22,26]. Likely, egress of virions is inhibited by cross-linking of newly formed virions to each other and to HA on the cell membrane [22]. These latter antibodies have for a long time been overlooked given that they cannot be detected with classical neutralization assays [25].

In contrast to these strain-specific anti-HA head neutralizing antibodies, some neutralizing antibodies directed against the HA head can neutralize different strains and even different



**Figure 2.** Mechanisms of neutralizing antibodies. ① Preventing interaction between virus and sialic acid receptor. ② Preventing fusion of viral and endosomal membrane. ③ Preventing release of newly formed viral particles. ④ Preventing cleavage of HA0 to HA1 and HA2 by membrane bound proteases.

subtypes of influenza virus [27–29]. Antibodies that are able to neutralize several strains of influenza virus are called broadly neutralizing antibodies (bNAbs). bNAbs directed against the HA head bind to conserved regions of this domain, for example the pocket of HA which binds sialic acid [10]. However, the epitope-binding region of an antibody is larger than this pocket and these antibodies will, therefore, usually also be in contact with variable parts of the HA molecule [18]. bNAbs against the HA head are rarely found, possibly because the HA evolved in such a way that the receptor-binding pocket, which needs to be conserved and is therefore vulnerable for immune recognition, is kept small and is surrounded by highly variable regions which allow immune escape [18,30].

## 2.2. Neutralizing antibodies targeting the HA stem

Neutralizing antibodies directed against the stem part of HA are far less commonly found after vaccination or exposure to virus than anti-HA head neutralizing antibodies [30]. The stem of HA, although more conserved, is generally less immunogenic than the head. One possible reason is that the bulky head of the HA protein impairs the accessibility of the stem for antibodies [31]. However, low amounts of anti-HA stem neutralizing antibodies can be detected in humans after infection by influenza viruses or after vaccination, with infection being more effective than vaccination in inducing this type of antibodies [30,32].

Antibodies against the HA stem interfere with fusion of the virus with the endosomal membrane following internalization of the virus by the cell and lowering of the endosomal pH [22,33]. For the fusion process to occur, a network of at least 3–5 neighboring HA proteins inserting their fusion peptides into the endosomal membrane is needed [34–36]. HA stem-directed

neutralizing antibodies have the ability to prevent the pH-induced exposure of the HA fusion peptide and thereby disrupt the formation of the network of HA proteins engaging with the endosomal membrane [24,33,37]. About 60% of the HA proteins has to be covered with antibodies to effectively prevent fusion of the viral and endosomal membrane [33].

Inhibition of fusion is the main effector mechanism of anti-HA stem-directed neutralizing antibodies in the protection against influenza virus, however, some of these antibodies are also able to inhibit the cleavage of HA0 [22]. HA0 is the precursor of HA1 and HA2 and cleavage of HA0 into HA1 and HA2 is needed to enable newly formed virions to successfully infect new host cells [38]. Proteases on the surface of the host cell are responsible for this cleavage [39]. Some HA stem neutralizing antibodies are able to bind HA0 close to the cleavage site on the surface of an infected host cell and can thereby prevent membrane-bound proteases from cleaving HA0 [22]. Moreover, HA stem neutralizing antibodies have been shown to be able to inhibit egress of newly produced viral particles [26]. The HA stem neutralizing antibodies can, thus, be protective by the inhibition of membrane fusion, the inhibition of HA0 cleavage, and inhibition of viral egress from the infected cells.

The breadth of cross-reactivity against different strains, subtypes, or types of influenza virus is dependent on the level of conservation of the epitope that these antibodies bind [10]. Most HA stem-specific bNAbs are able to bind to different subtypes of influenza virus within one of the two phylogenetic groups of influenza virus type A [24]. Anti HA stem bNAbs are most frequently found for group 1 viruses [10]. Only a few of the bNAbs directed against the HA stem are found to neutralize different subtypes of influenza within group 2 [40]. Some HA stem bNAbs have the



ability to bind different strains of both phylogenetic groups, however these are rarely found [10]. These antibodies have the ability to do this because they have multiple binding modalities, so they can bind multiple related structures on the HA-stem of different subtypes [10]. A bNAb that can bind to both different influenza virus type A strains as well as influenza virus type B strains has also been described [41].

Compared with the highly variable epitopes located in the HA head domain, the mutation rate of the HA stem region is rather low. Nevertheless, even in the case of stem-binding antibodies, we witness the phenomenon of influenza immune escape [42,43]. Amino acid changes allowing immune escape can result in loss of recognition by antibodies. For example, it has been described that the stem part of HA can undergo antigenic drift when HA stem directed bNAbs are present [44]. The drifted viruses regained their ability to infect host cells in the presence of bNAbs. Yet, it has also been shown that influenza A virus can escape antibody neutralization via mutations that do not alter antibody binding. Chai et al. described a mechanism by which mutations enhanced the fusion ability of the virus [43]. These mutations led to reduced binding of the antibodies at low pH, as encountered in the endosome after virus internalization. At the same time, the mutated HA could achieve its fusion-active conformation at higher pH than the wildtype variant thus extending the pH window under which fusion could take place. Thus, immune escape from bNAbs directed against the HA stem does occur. However, most of these mutations negatively affect the fitness of the virus and accordingly the importance of these immune escape mechanisms *in vivo* remains unclear and needs to be further investigated. Moreover, while single point mutations easily prevent binding of antibodies to the globular head of the HA molecule thus leading to immune escape, single point mutations in the stem region seem to only moderately reduce antibody binding and several mutations are necessary to achieve immune escape [45].

### 2.3. Antibodies targeting NA

The NA protein is important at different stages of the infection process. Due to its sialidase activity, NA allows the virus to reach the target cells by cleavage of sialic acids from respiratory tract mucins [46]. Also, NA makes budding of new virions possible by preventing virions to remain bound to sialic acid residues on the host cell. In addition, NA prevents aggregation of newly formed virions, caused by the interaction of the HA on one virion with sialylated glycans on a second one [46].

Antibodies targeting NA come into play when HA-binding antibodies are absent or are unable to prevent infection [47]. Immunity directed against the influenza virus NA has been shown to provide protection against viral infection in animal models [48–51]. Furthermore, several studies have demonstrated that NA-based immunity also correlates with protection from influenza virus infection and disease in humans [46,52]. Recently, Rajendran *et al.* assessed the breadth, functionality, and isotype/subtype usage of anti-NA antibodies in children, adults, and the elderly. They found that anti-NA titers increase with age, but the magnitude of the response is subtype

dependent and appears to be low for N1, whereas it is higher and cross-reactive for N2 and influenza B virus NA [46].

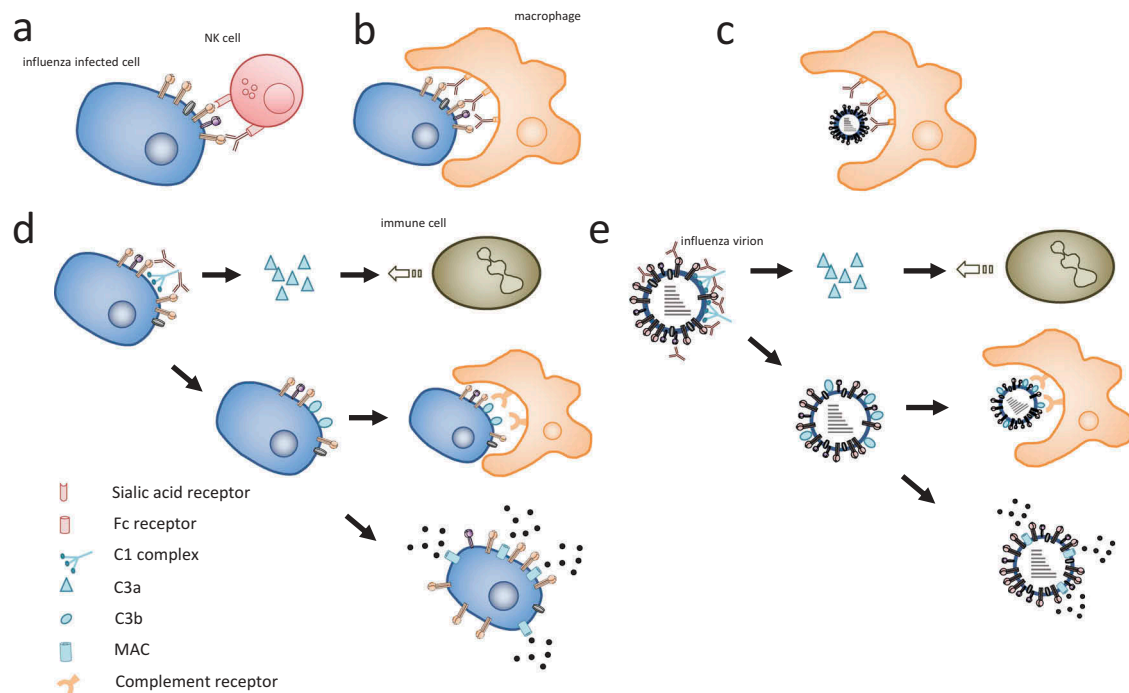
Recent findings demonstrate that influenza virus infection induces NA-reactive B cells to an equal or even higher level than HA-reactive B-cells. In contrast, current influenza vaccines poorly display key NA epitopes and consequently rarely induce NA-reactive B-cells [53]. Since NA-binding antibodies appear to be broadly reactive to virus strains which circulated in humans in the past, a vaccine eliciting broadly reactive antibodies against NA would be greatly beneficial [53]. Given the fact that many questions remain open regarding neuraminidase-based immunity, a neuraminidase focus group (NAAction) was formed at a Centers of Excellence for Influenza Research and Surveillance meeting at the National Institutes of Health in Bethesda, MD, to answer these questions and fill in the knowledge gaps in this field with the goal to implement better influenza vaccines [54].

### 3. Working mechanisms of non-neutralizing antibodies

Traditionally, the ability of antibodies to directly neutralize influenza virus was considered as the most important function in the protection against influenza virus. However, recent findings show the importance of other antibody-mediated effector mechanisms, which are also contributing to protection against influenza virus but have so far been largely overlooked [55,56]. Given the fact that non-neutralizing antibodies often target more conserved epitopes of influenza virus proteins they may be of particular importance for the development of vaccines with broadly protective function. Three effector mechanisms that can be induced by non-neutralizing antibodies are known to contribute to protection against influenza virus. These effector mechanisms are antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent complement deposition (ADCD) (Figure 3) [10,11].

In contrast to neutralizing antibodies which exert their function via the variable part of the antibody molecule alone, the effect of non-neutralizing antibodies is dependent on both the variable region and the Fc region of the antibody [57]. The Fc region has the ability to interact with other components of the immune system, at least when the antibody is bound to an antigen. Five classes of antibodies can be distinguished based on the structure of their Fc region, namely, IgA, IgD, IgE, IgG, and IgM [58]; among these IgG and IgM are particularly important for non-neutralizing antibody effector functions. IgG can be further subdivided into four subclasses (IgG1, IgG2, IgG3, and IgG4 in humans), which differ in their Fc region and accordingly in their biological characteristics. IgG3 is considered as the IgG antibody subclass with highest functional potency when compared to the other IgG subclasses [59]. Furthermore, according to recent results not only the Fc region itself but also its glycosylation status is important for the functional potency of non-neutralizing antibodies [11,50].

Non-neutralizing antibodies can work by binding of the Fc region to specific Fc receptors present on a variety of immune cells including NK cells, macrophages, and neutrophils [57]. After binding, these immune cells can be activated and help in the



**Figure 3.** Mechanisms of non-neutralizing antibodies. (a) Antibody-dependent cellular cytotoxicity (ADCC): Influenza-specific IgG binds to the viral proteins on the cell surface, thus opsonizing infected cells. Subsequently, Fc gamma receptor IIIa (FcγRIIIa), expressed by innate effector cells like NK cells, binds to the Fc region of the bound IgG while at the same time sialic acid receptors on the NK cells engage HA on the infected cell. Thus activated NK cells release cytotoxic factors that cause the death of the infected cell as well as antiviral cytokines (IFN $\gamma$ , TNF $\alpha$ ) and chemokines [70]. (b) Antibody-dependent cellular phagocytosis (ADCP) of infected cells and (c) viral particles. After opsonization of a microbe or an infected cell by antibodies, phagocytes like monocytes, macrophages, neutrophils, and dendritic cells recognize the antibodies that are bound to the foreign particles with their antibody receptors. Phagocytes will then engulf and destroy the opsonized cells or viral particles [72,78]. (d) antibody-dependent complement deposition (ADCD); complement-dependent lysis (CDL) and complement-dependent opsonophagocytosis of infected cells and (e) viral particles; Following the classic pathway, complement factor C1q binds to antibody opsonized viral particles or infected cells. In the subsequent cascade of events, C1q deposition enables the generation of anaphylatoxins like C3a and C5a, formation of opsonins like C3b and C5b which remain bound to the membrane and stimulate phagocytosis, and ultimately formation of a membrane attack complex (MAC) which can destroy virus particles [84,86].

protection against influenza virus. Six different Fc receptors, either activating (FcγRI, IIA, IIC, IIIA and IIIB) or inhibitory (FcγRIIB1/B2), have been described in humans [60]. Alternatively, non-neutralizing antibodies can activate the complement system via the classical pathway. Thus, non-neutralizing antibodies have an important role in connecting innate and adaptive immunity mechanisms [10].

### 3.1. Antibody-dependent cellular cytotoxicity (ADCC)

Cells infected with influenza virus present viral proteins, mostly HA and NA, on their surface because new virions are being formed and will eventually bud from the cell membrane. Influenza-specific IgG can bind to the viral proteins on the cell surface, thus opsonizing infected cells. Subsequently, Fc gamma receptor IIIa (FcγRIIIa), expressed by innate effector cells like natural killer (NK) cells, monocytes and macrophages, binds to the Fc region of the bound IgG. Crosslinking of FcγRIIIa molecules on the surface of the effector cell leads to phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) and subsequent activation of a Ca<sup>2+</sup>-dependent signaling pathway. Following these events, NK cells release cytotoxic factors that cause the death of the infected cell (perforin and proteases known as granzymes) as well as the production of antiviral cytokines like IFN $\gamma$ , TNF $\alpha$

and chemokines. These antiviral cytokines and chemokines contribute further to a decrease of viral replication [17].

Influenza ADCC is epitope-dependent as demonstrated by DiLillo et al. in 2014 when they examined the importance of epitope localization for influenza-specific ADCC using a panel of monoclonal Abs (mAbs) [56]. The authors showed that bNAbs targeting the highly conserved HA stem protected mice from lethal influenza challenge with H1N1 viruses through a mechanism that involved Fc-FcγR interactions. In contrast, strain-specific mAbs against the variable head domain of HA were equally protective in the presence or absence of FcγR interactions, which suggested that they provided protection in an FcγR-independent manner [16,56]. The group further demonstrated that ADCC was necessary for *in vivo* protection and that the antiviral activity of HA stem bNAbs was mediated through activating FcγRs [56].

In a subsequent study, DiLillo et al. screened a panel of 13 anti-HA mAbs (bNAbs and non-neutralizing Abs, against both the stalk and head domains) and showed that all classes of broadly binding anti-HA mAbs required Fc-FcγR interactions to provide protection *in vivo* [61]. Interestingly, only antibodies targeting the HA stem but not antibodies targeting the HA head were found capable of mediating ADCC. In fact, anti-HA-head antibodies when mixed with anti-stem antibodies appeared to hamper ADCC induced by the latter [62]. It was later demonstrated that the receptor-binding domain of HA on infected cells is required to bind to sialic acid expressed on

the surface of effector cells to optimize effector cell activation in the context of ADCC [62,63]. In particular, broadly reactive mAbs seem to require two molecular contacts, possibly to stabilize the immunologic synapse and fully induce the antiviral activity of the innate effector cell. The first contact is the interaction between the Fc of a mAb bound to HA with the FcγR of the effector cell, and the second contact is the interaction between the HA on the infected cell and its sialic acid receptor on the effector cell [63].

It is very likely that in the natural situation there is not only one type of antibody but instead an ensemble of different types of antibodies against influenza antigens. He et al. showed that interactions among antibodies that bind viral glycoproteins of varying specificities regulate the extent to which ADCC is induced [64]. They also show that interactions among antibodies that bind to discrete epitopes on the same antigen can influence the induction of Fc-dependent effector functions. Their observations imply that when sufficient neutralizing antibodies are present, ADCC is inhibited, and neutralization is the primary mode of protection. However, when neutralizing antibodies are absent or limited, bnAbs targeting the HA stalk domain engage effector cells to stimulate ADCC and thereby limit the spread of infection.

Antibodies to conserved, internal viral proteins, such as NP, also seem to contribute to protection against influenza [65]. ADCC-mediating Abs to internal viral proteins such as NP and M1/2 are induced by influenza infection and vaccination. These conserved internal proteins can be found on the surface of infected cells *in vitro* and several lines of evidence support the fact that antibodies against NP and M2 are involved in ADCC [66]. ADCC-mediating antibodies to NP have recently been shown to be induced through vaccination of children with seasonal inactivated influenza vaccine [67]. Moreover, recent work by Vandervan et al. demonstrates that healthy and influenza infected human individuals have anti-NP antibodies that can cross-link FcγRIIIa and activate human NK cells [68]. Finally, it has been found in ADCC assays that sera from healthy humans containing anti-NP antibodies can induce robust NK cell activation against NP-expressing and virus-infected cells [67].

Another conserved influenza protein found on the surface of infected cells is M2. Antibodies against this protein were found to be protective in mice. It was demonstrated that passive immunotherapy with a fully human mAb targeting the M2 protein in mice resulted in significant protection from lethal infection. Importantly, ADCC and complement were proven to be required for non-neutralizing anti-M2 antibody-mediated protection *in vivo* [69]. Another study showed that a human mAb targeting the M2 protein (Ab1-10) was capable of activating NK cells and mediating ADCC *in vitro*. In particular, it was shown that the above-mentioned mAb is capable of mediating ADCC against cells transfected with the M2 protein and also against influenza-infected cells [70].

### 3.2. Antibody-dependent cellular phagocytosis (ADCP)

Phagocytosis is an important immunological process in which phagocytes ingest microbes and infected cells [71]. One specific form of phagocytosis is ADCP. The first step in ADCP is

opsonization of a microbe or an infected cell by antibodies [71]. After opsonization, phagocytes recognize the antibodies that are bound to the foreign particles with their antibody receptors, in particular the Fcγ receptors CD32 (FcγRIIA) and CD64 (FcγRIA) and the Fcα receptor CD89 [72]. The most important phagocytes involved in ADCP are monocytes, macrophages, neutrophils, and dendritic cells [71]. When the phagocytes recognize an opsonized viral particle or opsonized infected cell with their antibody receptor, phagocytes will engulf them in a phagosome [73]. Phagosomes mature and then fuse with lysosomes. These lysosomes contain different enzymes and superoxide that are able to destroy the content of the phagolysosomes [74]. In this way not only foreign particles but also opsonized infected cells can be destroyed.

ADCP is one of the important antibody-induced effector mechanisms that contribute to protection against influenza. Already in 2001 Huber et al. investigated the role of opsonophagocytosis in protection against lethal influenza virus infection using FcγR  $-/-$  mice [75]. They showed that FcγR  $-/-$  mice were highly susceptible to influenza infection, even in the presence of anti-influenza Abs from immune FcγR  $+/+$  mice. NK cells were not required for Ab-mediated protection, but macrophages from FcγR  $+/+$  mice could actively take up opsonized virus particles indicating that Fc receptor-mediated phagocytosis can be of major importance in clearing influenza virus infections. Further indications for a role of ADCP in the defense against influenza came from studies in which live influenza A virus particles and influenza protein-coated fluorescent beads were opsonized with antibodies and their uptake into a monocytic cell line was measured. Both serum IgG from healthy adults and from pooled intravenous immunoglobulin (IVIG) preparations was found to contain antibodies capable of mediating ADCP of beads coated with HA from multiple virus subtypes [76]. It is likely, as the authors suggest, that the numerous exposures of human adults to influenza (through previous infection or vaccination) cause low levels of cross-reactive antibodies capable of mediating ADCP, which are boosted by subsequent heterologous infections [77]. These results did not prove, although, that antibodies can indeed confer protection against influenza infection by means of ADCP. Evidence for the protective potential of ADCP-inducing antibodies, at least in the mouse model, comes from an *in vivo* study by Dunand et al. These authors showed that some broadly cross-reactive non-neutralizing mAbs protected mice through Fc-mediated effector cell recruitment and in particular they did so exclusively through ADCP and not through ADCC or ADCL [55]. Several antibodies with different specificities have the ability to mediate ADCP among which even bnAbs directed against the HA stem [55,78,79].

Digging deeper in the analysis of influenza-specific ADCP, it was demonstrated that alveolar macrophages (AMφ) are critically involved in ADCP induced by murine as well as human monoclonal Abs *in vivo* and are necessary for conferring optimal protection against homologous and heterologous virus challenge [79]. Both non-neutralizing Abs and bnAbs can mediate ADCP of opsonized virus and are able to activate AMφ to establish a pro-inflammatory environment [79]. Interestingly, Wong et al. recently documented in mice that with aging, AMφ exhibit an impaired ability to limit lung damage during influenza viral infection [80]. It is tempting to



speculate that impairment of ADCP is one reason for this phenomenon.

Apart from AM $\phi$ , other innate effector cell populations can be involved in ADCP-mediated protection against influenza. Mullarkey et al. focused on neutrophils, which are the most abundant subset of blood leukocytes and which express both activating and inhibitory Fc $\gamma$ Rs [78]. The group analyzed the interplay between HA stem-specific IgG, Fc-Fc $\gamma$ R engagement, and neutrophil effector functions. They demonstrate that human and mouse monoclonal HA stem-specific IgG antibodies are able to induce the production of reactive oxygen species (ROS) by neutrophils and that this phenomenon is dependent on FcR engagement and phagocytosis. They also showed that this was not the case for HA head-specific antibodies, confirming once again how the ability of HA stem-specific antibodies to mediate Fc-Fc $\gamma$ R receptor engagement is epitope-dependent [78].

### 3.3. Antibody-dependent complement deposition (ADCD)

The complement system is made of soluble and membrane proteins, which are found ubiquitously in the blood and tissues of mammals. These proteins interact with each other and with other components of the immune system building up a series of effector proteins that contribute to the elimination of various pathogens, including influenza virus. Complement activation can occur through three pathways, which are called the classical, lectin, and alternative pathways, and it represents an important link between innate and adaptive immunity [81]. Noteworthy, according to textbook knowledge, only the classical pathway depends on the presence of antibodies against the pathogen. The central events in complement activation are proteolysis of the complement protein C3 into C3a and C3b, and later proteolysis of C5 into C5a and C5b. C3a and C5a serve as 'anaphylatoxins' and help in recruitment of leukocytes. C3b and C5b are opsonins which remain bound to the pathogen and enhance phagocytosis by neutrophils and macrophages. In addition, C5b enables deposition of additional components of the complement system which eventually results in formation of the membrane attack complex [11,82].

The fact that human sera contain antibodies capable of neutralizing influenza virus by engaging the classical pathway of the complement system was shown as early as 1982 by Beebe et al. [83]. Later studies confirmed that IgG and IgM bound to influenza virions activate the classical pathway of complement (C1) resulting in neutralization of infectivity [84,85]. The alternative pathway was found to be also activated, yet, was on its own not capable of neutralizing the virus. Interestingly, activation of the alternative pathway also required IgG suggesting an interplay between the classical and the alternative pathways [83]. In-line with these results, a later study demonstrated that protection of mice from influenza A(H1N1)pdm09 infection required an intact classical and alternative pathways, both requiring IgG for activating influenza neutralizing functions. Synergy between the two pathways was found to be critical to contain the infection [85].

Further investigations gave a somewhat complicated picture. Influenza virus H1N1pdm09 appeared to be resistant to lectin pathway-mediated neutralization as well as to alternative pathway-mediated neutralization in the absence of antibodies. In contrast, seasonal influenza A H3N2 virus was found to be susceptible to alternative pathway-mediated neutralization even in the absence of antibodies. The difference between the strains could be attributed to the fact that on H3N2 virus C3b deposition could take place through covalent linkage of C3b to HA and/or NA while HA/NA of H1N1pdm09 virus did not support C3b binding [85].

The complement system is not only able to neutralize virus particles but also involved in the lysis of infected cells in humans. Vaccination with standard trivalent-inactivated influenza vaccine was found to increase levels of antibodies capable of mediating complement-dependent lysis (CDL) of influenza virus-infected cells *in vitro*, though fold increases were moderate [86]. Although direct proof is lacking, these antibodies can potentially contribute to clearance of both viral particles and infected cells *in vivo*. Further analysis of infection or vaccination-induced human antibodies with the ability to induce CDL revealed that most of these antibodies also have virus-neutralizing capacity [87]. However, not all neutralizing antibodies were able to mediate CDL. Both HA head neutralizing antibodies and HA stem neutralizing antibodies were found to be able to mediate CDL. The HA stem neutralizing antibodies were, however, more cross-reactive and, thus, were able to induce CDL of different strains of influenza virus.

Many viruses make use of complement evasion strategies by encoding proteins that bind and inhibit or sequester complement components. Evidence exists that the matrix (M1) protein prevents complement-mediated neutralization of influenza virus *in vitro* by binding C1q and blocking the interaction between C1q and IgG [88].

## 4. Conclusion

We reviewed here the main effector mechanisms of antibodies directed against influenza virus. Antibodies can confer protection against influenza through Fc-independent or Fc-dependent mechanisms. Fc independent antibodies directly neutralize the virus by interfering with the process of virus entry, fusion, or egress. Mainly antibodies against HA and NA are involved in direct neutralization of the virus [24]. Since most directly neutralizing antibodies target highly variable epitopes, they are usually strain-specific. Fc-dependent antibodies do not act via direct neutralization but rather activate cell-dependent or complement-dependent effector mechanisms [17]. Non-neutralizing antibodies can interact through their Fc part with Fc $\gamma$ R located on the membrane of immune effector cells thus causing ADCC or ADCP, or can activate the complement system via the classical pathway. Antibodies that act through these mechanisms can be directed against HA, NA, M2, or NP. Therefore, they can target variable as well as conserved epitopes of influenza viruses [67,70].

## 5. Expert commentary

Fostered by research initiated after the threat of an H5N1 pandemic in the early 2000s and the 2009 H1N1 pandemic, our knowledge on antibody-mediated immune responses to influenza has increased enormously in recent years. Three observations which are of particular importance in this context are described in the following.

The first observation was the detection of broadly neutralizing antibodies, thus antibodies which could not only neutralize one influenza virus strain but several. Indeed, by now, antibodies have been detected which can neutralize several influenza A subtypes, all subtypes within one of the two phylogenetic groups of influenza A viruses, viruses of both phylogenetic groups or provide protection against all influenza A and B viruses [18,53,89]. Although these antibodies are found infrequently and in low amounts after infection or vaccination, their existence has encouraged the search for vaccines capable of inducing such bNAbs. Many of the bNAbs were found to target the HA stem region which is much more conserved than the HA head region. Approaches to focus vaccine-induced antibody responses on the stem include sequential immunization with vaccines containing different HA molecules, sequential immunization with chimeric HA molecules carrying different irrelevant heads and the same stem, and mini-HA constructs consisting of only the HA stem region (for a recent review see [90]). Several of these approaches are currently tested in clinical trial, and the results are awaited eagerly.

The second important observation was that influenza-specific antibodies do not necessarily have to interfere with the infection process as such but can rather use other effector mechanisms like ADCC, ADCP, or ADCD to counteract viral infection. Non-neutralizing antibodies had earlier been largely overlooked, partly because the assays needed to detect them require specific material (e.g. NK cells, macrophages) and are tedious and often not well reproducible. Influenza-specific antibodies which work via ADCC, ADCP, or ADCD can target different viral proteins including conserved ones like NP and M2 and can thus be highly cross-reactive. This opens new possibilities for the design of broadly protective vaccines. In this context, it is very important to realize that ADCC-, ADCP-, and ADCD-exploiting antibodies need to interact via their Fc part with Fc receptors on effector cells or with complement. Accordingly, the class and subclass of the vaccine-induced antibodies will become important as it will determine their biological function. In particular, IgG3 should be induced as this antibody subclass has high affinity to activating Fcγ receptors and high complement-fixing activity [57,59].

The third observation is that the B-cell response to influenza infection but also to vaccination consists to a rather large extent of pre-existing cross-reactive antibodies rather than of newly induced strain-specific antibodies [21,54,91]. Thus, rather than activating naïve B-cells, infection and vaccination re-activate memory B-cells which were induced by previous encounter of influenza antigens. The phenomenon has been addressed as 'original antigenic sin' thus emphasizing a possible detrimental role of pre-existing immunity. Yet, recently the term 'antigenic seniority' has been coined to underline

that pre-existing immunity can have both positive and negative effects on the response to vaccination [77]. In any case, the effects of pre-existing immunity imply that infection and vaccination history will have a major impact on the response to any new influenza vaccination. While pre-existing memory B-cells might be disadvantageous for induction of neutralizing antibodies to the receptor binding site of new variants of HA, they may be highly advantageous to achieve effective antibody responses against conserved influenza antigens; antibodies which work via neutralization of the HA stem or via non-neutralizing effector mechanisms. A better knowledge of the role of pre-existing influenza-specific immunity on the response to vaccination in its entire breadth may allow for the design of vaccines which optimally exploit pre-existing immunity. Such a vaccine could, for example, be tailored to specific target groups, in particular young children and the elderly. Interestingly, already in 1960 Thomas Francis Jr suggested that OAS could be exploited to our advantage by vaccinating children early in life with several influenza strains so that natural infections later in life would serve to broaden the vaccine-generated immunity [7].

## 6. Five-year view

The insight that antibodies not detected in current neutralization assays and non-neutralizing antibodies are able to confer protection against influenza will stimulate the development of new assays with which these antibodies can be reliably measured. Application of these assays will allow us to determine the levels of these antibodies in the general population and how these levels change upon vaccination. Non-neutralizing and non-classical neutralizing antibodies will be further studied and their targets and mechanisms of action investigated. Eventually, we will be able to understand the role of these antibodies as well as their interplay in providing protection. Identification of the most frequent or most potent antibodies prevailing in absence of neutralizing antibodies will allow designing vaccines capable of inducing these antibodies and will get us closer to broadly protective vaccine. The concept of antigenic seniority and the impact that this phenomenon has on the boosting/generation of influenza-specific antibodies will be further investigated. Eventually, integration of knowledge in both fields, non-neutralizing or non-classical neutralizing antibodies and the role of pre-existing antibody responses, will allow us to design novel immunization strategies capable of providing broad and strong protection and optimized for different vulnerable groups of the general population.

## Key issues

- The ability of a vaccine to elicit antibodies is currently mainly measured by means of hemagglutination inhibition (HAI) or microneutralization (MN) assays. This practice has resulted in overlooking of non-neutralizing antibodies and antibodies, which neutralize in a non-classical way.
- Classic neutralizing antibodies interfere with virus binding to its cellular receptor. Other ways of neutralization are

inhibition of fusion, cross-linking of budding virions at the cell surface and prevention of NA-dependent release of newly formed virus particles.

- Non-neutralizing antibodies have been demonstrated to be able to confer protection against influenza virus via ADCC, ADCP, and ADCD. These antibodies should, therefore, be determined upon vaccination in addition to HAI/MN antibodies and their role in protection should be further investigated.
- Future research is needed to unravel the mystery behind the impact of pre-existing immunity on the boosting/generation of influenza-specific antibodies following vaccination. Acquired knowledge from these studies will allow us to eventually exploit pre-existing immunity to our advantage.

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